MICROBIOLOGICAL PROBLEMS IN STRIP MINE AREAS: RELATIONSHIP TO THE METABOLISM OF *THIOBACILLUS FERROOXIDANS*[1, 2]

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ABSTRACT

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The role of the iron oxidizing bacteria in contributing to acid mine drainage is considered relative to the metabolism of the organism. To study acid production an *in vitro* system was developed using isolated cell envelopes from *Thiobacillus ferrooxidans*, which retained the ability to oxidize Fe^{2+}. An oxygen electrode was used to follow oxygen depletion as Fe^{2+} was oxidized. The kinetics of Fe^{2+} oxidization was analyzed over a temperature range of 15° to 60° C and temperature effects were plotted as Arrhenius plots and K_m's and V_{max}'s were determined at different temperatures.

Activation energies of 4.5 kcal/mole for cytochrome oxidase and 11.4 kcal/mole for cytochrome *c* reductase were noted for the two enzymes involved in Fe^{2+} oxidation. The relative binding affinity of Fe^{2+} (K_m) was relatively stable to temperature changes but reaction rates (V_{max}) did increase as expected.

The power of the microbe is a continuously misunderstood phenomenon in sanitary biology. Because microbes are inconspicuous, they are often overlooked as agents of environmental problems. As a general microbiologist, I have always accepted a doctrine advanced by microbial scholars—never underestimate the power of the microbe. Also, I have learned that within the field of microbial ecology the concept of chemoautotrophy is extremely vital to one's understanding of how the microbe contributes to the movements of the earth and the various elemental cycles of nature. This concept should not be strange to this audience, for within this concept lies an understanding of the biological problems we are discussing during the symposium.

Chemoautotrophic bacteria are those that obtain their energy necessary for growth and carbon assimilation from the oxidation of reduced sulfur, and iron compounds, iron metals, ammonium ion, nitrite ion and hydrogen. The bulk of their carbon for existence comes from CO_2 assimilation.

Of immediate attention and interest to this group are the iron and sulfur oxidizing bacteria which are very much concerned with acid mine drainage, the mining of copper and uranium and the displacements of large buildings. Time does not permit discussion of the latter two problems, but they are becoming more and more important as man is faced with energy depletion and construction needs.

To focus upon our immediate problem of acid mine drainage and the bacterium *Thiobacillus ferrooxidans*, a fundamental reference to Singer and Stumm (1970) is important. These authors relate the following conditions:

$$(1)\quad FeS_2 \xrightarrow{(+O_2)} Fe^{2+} + S\text{—compounds}$$

$$(2)\quad \rightarrow Fe^{2+} + O_2 \xrightarrow[\text{catalyst}]{\text{bacterium}} Fe^{3+}$$

$$Fe^{3+} + FeS_2 \longrightarrow Fe^{2+} + SO_4^{2-}$$

The equations are sequential reactions initiated by (1) the oxidation or dissociation of pyrite in which Fe^{2+} is released and (2) a cyclic process where ferrous iron is oxidized to ferric via oxygen in

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conjunction with a biological catalyst. The rate-limiting reaction is catalyzed by an acidophilic bacterium. The optimum pH of the reaction is around 2.5 to 3.5; Fe^{2+} is continuously regenerated by the reduction of Fe^{3+} by pyrite.

The environment contains many other examples of microbial-mineral interactions. Consider the following (Silverman and Ehrlich, 1974):

> "All living materials cause small scale transformations of trace elements necessary for enzyme activation and function. Microorganisms can by either direct or indirect action transform relatively large amounts of mineral matter sufficiently to influence its geological distribution. They do this via certain enzymatic processes involving:
>
> *a*. Redox reactions (figure 1).
>
> *b*. Digestion of metal complexes. Some inorganic ions in aqueous solution, particularly ions of iron, copper, maganese, zinc, calcium and magnesium from chelates or complexes with certain organic chemicals. Complexing agents generally stabilize inorganic ions in solution. Many bacteria can use the complexing agent as carbon or nitrogen sources thereby freeing complex inorganic ions. These can precipitate as water insoluble hydroxides or salts at the appropriate pH and Eh.
>
> Non-enzymatic reactions can also influence the transformations of mineral matter.
>
> *a*. Interactions with metabolic products. Products as H_2S, H_2CO_3, HNO_3, H_2SO_4 react nonbiologically with dissolved inorganic matter.
>
> *b*. Absorption of mineral matter to cell surfaces.
>
> Sheath bacteria carry out this function."

We made the assumption that the organism which acts as a catalyst in the field is going to perform the same way in the laboratory. Consequently, laboratory studies were devoted to the cultural, physiological and biochemical investigation of this bacterium.

This work is focused upon the mechanism of action of the biologically catalyzed oxidation of ferrous iron.

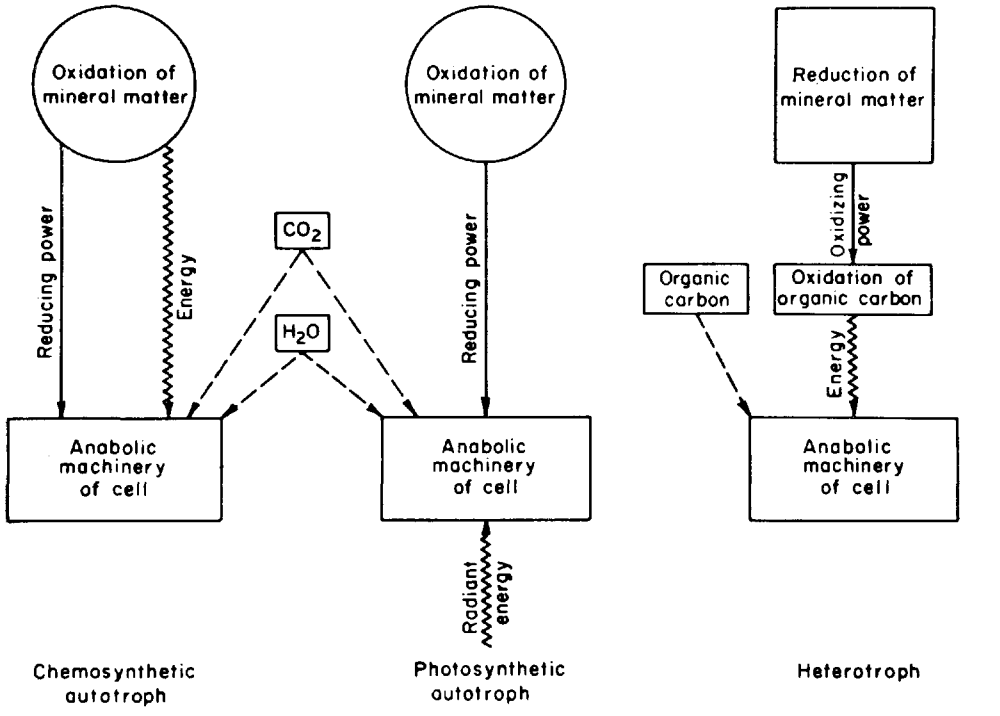


FIGURE 1. Schematic drawing (Silverman and Ehrlich, 1964), showing oxidation and reduction of mineral matter.

Figure 2 shows an electron micrograph of the catalytic organism *T. ferrooxidans*, isolated initially from a Pennsylvanian acid stream. It is in the inner layer

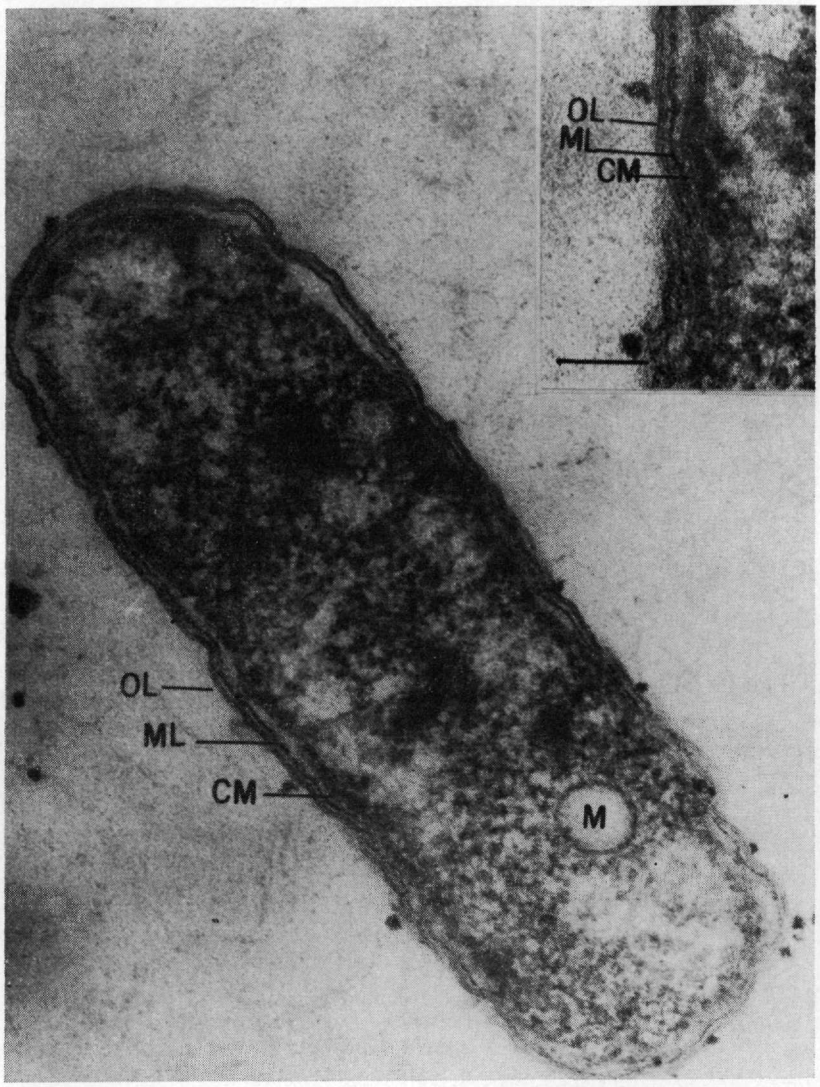


FIGURE 2. Electron micrograph of *T. ferrooxidans* showing the layered envelope components. OL, outer layer; ML, middle layer; CM, cytoplasmic membrane. 24, 300×.

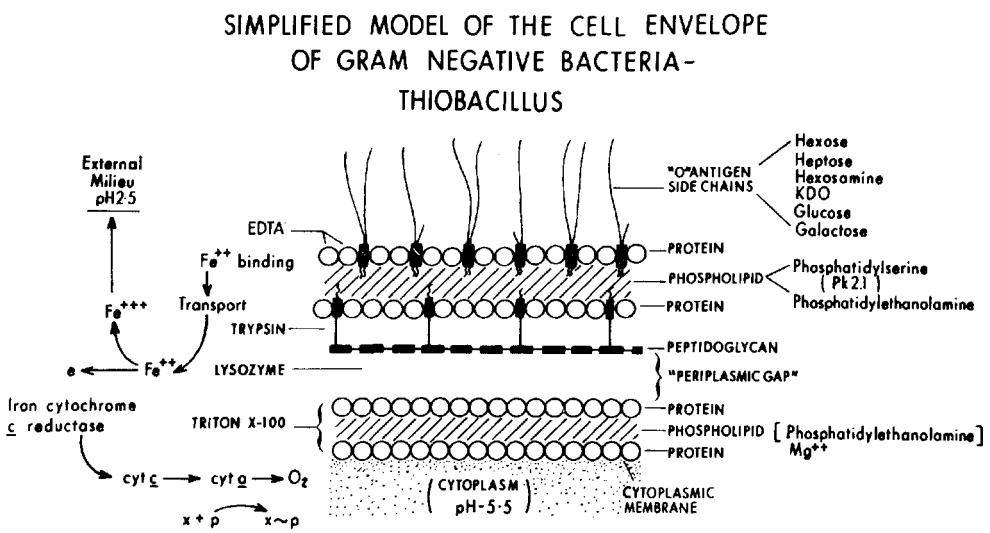


FIGURE 3. Model of a typical cell envelope of a gram-negative bacterium, *T. ferrooxidans*. Iron oxidation occurs in the cytoplasmic membrane of the bacterium.

(CM) where ferrous iron oxidation occurs. Further, within this narrow zone a micro-environment exists which separates an acid milieu (pH 2.5) from the cell cytosol (pH about 6.0).

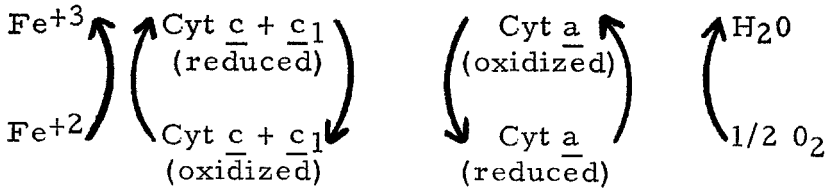
Figure 3 shows a typical model of the envelope of *T. ferrooxidans*, based on what is known of the cell envelope of gram-negative bacteria. Costerton *et al.* (1974) offer an excellent review of an in-depth appraisal of the cell envelope of gram-negative bacteria. The left side of the figure outlines those reactions which this paper will deal with, and more specifically, that portion of the figure which describes the loss of the electron from Fe^{2+} oxidation. Diagrammatically this can be described as:

Energy in the form of ATP is generated through the electron transport system. Noteworthy is the observation from the model that the electron transport system resides in the cytoplasmic membrane of the cell, and that it is one of the simplest electron transport systems known to bacteria. Lundgren *et al.* (1974) have published an extensive review of the ion oxidizing bacteria and their electron transport systems.

The electron transport system of *T. ferrooxidans* was investigated with clean cell envelope preparations. Figure 4 is an electron micrograph of cell envelopes prepared from intact cells. On the basis of cell protein, the envelopes contain about 40% of the iron oxidation activity

$$4\ FeSO_4 + O_2 + 2\ H_2SO_4 \longrightarrow Fe_2(SO_4)_3 + 2\ H_2O$$

or as



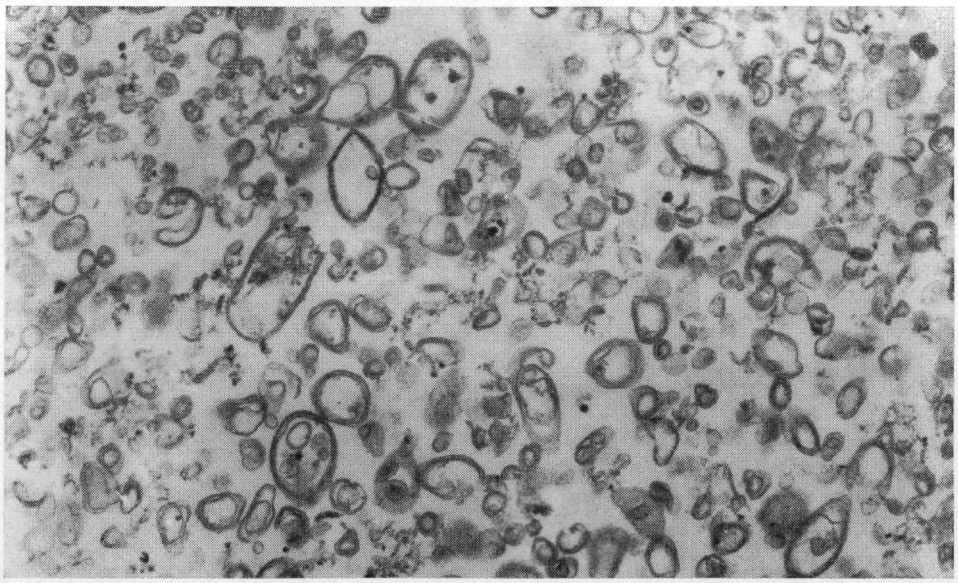


FIGURE 4. Electron micrograph of a cell envelope preparation isolated as described in the text. The envelope contains the "iron oxidase" enzymes. The three layers of the envelope are the same as those shown in the insert of figure 2.

of intact cells. Bodo and Lundgren (1974) have described the isolation as well as the application of the cell envelopes to the study of iron oxidation.

MATERIALS AND METHODS

Organism and culture procedures. T. ferrooxidans was grown on the standard 9K medium in 16-liter Nalgene carboys under forced aeration. The 9K medium was adjusted to pH 3.2 prior to inoculation, with H_2SO_4 and one ml of a 15% (w/v) cell suspension used as an inoculum. Carboys were aerated and maintained in a 30°C incubator.

Cells were harvested in a Sharples centrifuge after determining the absence of Fe^{2+} iron in the culture medium by adding, 2,2' dipyridyl to a sample of the culture and noting the absence of a red color. Fe^{+2} was completely oxidized by 60–70 hr. The collected cell paste was suspended in 40–80 ml of acidified (pH 3.0 with H_2SO_4) glass distilled water, and the accompanying ferric iron precipitates removed by low speed centrifugation (750 rpm) in a refrigerated RC2–B Sorvall centrifuge equipped with a SS–34 rotor. The cell suspension was decanted into clean 50-ml centrifuge tubes and centrifuged at 17,500 rpm for 15 min. The supernatant was discarded, and the cell pellet suspended and centrifuged at 17,500 rpm. The washing step was repeated three to four times until no iron (Fe^{3+} or Fe^{2+}) was detected in the supernatant. The final cell suspension was made up to a 20% (w/v) stock with acid water.

Preparation of crude envelopes. Fifteen ml of the 20% suspension of *T. ferrooxidans* were diluted to approximately 40 ml with distilled water, and centrifuged at 17,500 rpm for 15 min. as described. The cell pellet was suspended in 20 ml, 0.01 M PO_4^{3-} buffer (pH 7.0) and stored at 4°C overnight. The cell suspension was diluted to 40 ml with distilled water and centrifuged at 17,500 rpm for 15 min. The supernatant was discarded and the pellet suspended in 40 ml of glass distilled water and centrifuged as described earlier. The supernatant was discarded and the cell pellet suspended to a final volume of 20 ml. The cell suspension was passed 3 times through an Aminco Power driven French Pressure Cell Press at a gage pressure of 20,000, and the lysate collected. Whole cells were removed from the lysate by centrifuging at 7,500 rpm for 10 min; the supernatant was passed through a 0.45μm Millipore filter and 1 mM $MgSO_4$ added to the filtrate. The filtrate was centrifuged at 40,000 rpm for 1 hr in an International B–60 preparative ultracentrifuge equipped with an A–211 rotor. The resulting pellet was suspended in 0.01 M PO_4^{3-} (pH 7.0) buffer containing 1 mM $MgSO_4$ to a protein concentration of 20 to 25 mg protein/ml. This preparation was designated the cell envelope fraction.

Assays. Iron oxidation by both intact cells and cell envelopes of *T. ferrooxidans* was assayed by measuring oxygen uptake with a Clark oxygen electrode. The components added to a water cooled, 2 ml volume reaction vessel containing the electrode were: either 0.1 ml of a 1% (w/v) suspension of whole cells or 0.1 ml of the cell envelope preparation (20–25 mg protein/ml); 1.8 ml of 0.02 M B-alanine-SO_4^{2-} buffer (pH 3.5); 0.1 ml of 0.2 M $FeSO_4 \cdot 7H_2O$ in

pH 2.5, H_2SO_4. The iron initiated the reaction and was delivered with a microliter syringe. All assays were normally conducted at 28°C and were agitated by means of a magnetic stirring bar. Auto-oxidation of $FeSO_4$ was checked during each set of assays by omitting whole cells or envelopes and adding 0.1 ml of the buffer in its place. Results were always expressed as averages of at least these separate experiments. Different cell batches were always used to check results.

For studying temperature effect upon iron oxidation, a temperature range of 15 to 60°C was used. Constant temperatures were achieved by using a circulating water bath. Temperature effects on iron oxidation were plotted as Arrhenius plots. K_m's for the affinity of iron and the V_{max} of iron oxidation were also determined for cell envelopes exposed to varying temperatures.

RESULTS

The linearity of O_2 uptake with increasing concentrations of cell envelopes extended over an envelope protein concentration range of 2–10 mg of protein. The pH activity curve for the cell envelopes showed an optimum at 2.5.

The oxidation of ferrous iron and concomitant uptake of oxygen involves a two component electron transport system-cytochrome *c* reductase and cytochrome oxidase. The present understanding of the characteristics of these enzymes is summarized in table 1. The data comes mostly from the research of Blaylock and Nason (1963) and Din *et al.*, (1967).

Using metabolic inhibitors and the envelope preparation, and measuring oxygen uptake involving the intact electron transport chain, other information has been obtained. Table 2 shows that iron oxidation was inhibited by cyanide and azide, both inhibitors of cytochrome oxidase, and also by silver nitrate and uranyl ions; these ions can affect electron

TABLE 1

Characteristics of Iron Cytochrome C Reductase and Cytochrome Oxidase.

Enzyme
Iron Cytochrome *c* Reductase (partially purified)
1. Closely associated with membranes; difficult to solubilize.
2. Composed of two components: protein and RNA. High stability to heat, proteolytic enzymes and RNAase.
3. High salt conc. causes an inactivation by dissociation of the two components.
4. Purified enzyme contains one single protein, MW 30,000; holoenzyme, MW = 110,000.
5. One atom of Fe^{++} associated with the protein portion.
6. K_m of 1×10^{-3} M for Fe^{++}; 9.5×10^{-5} M for Cyt *c*.
7. No sulfhydryl group in enzyme.
8. No flavin compound in enzyme action.
9. pH optimum, 5.7–7.0.
Cytochrome Oxidase (crude preparation)
1. pH optimum 4.3–4.7
2. K_m for reduced Cyt *c*, 4.6×10^{-6} M
3. heat sensitive
4. inhibited by KCN, NaN_3, Na_2S

TABLE 2

*Effect of Inhibitors on Iron Oxidation by Cell Envelopes.**

Compound	Final concentration (M)	Inhibition %
NaN_3	1×10^{-4}	100
NaCN	1×10^{-4}	100
$AgNO_3$	1×10^{-3}	85
Uranyl acetate	1×10^{-3}	55
Antimycin A	2×10^{-4}	0
4-Hydroxy-2-n-heptylquinoline N-oxide	1×10^{-3}	0
Sodium Amytal	1×10^{-3}	0
Atabrine	1×10^{-3}	0
Iodoacetamide	1×10^{-3}	0
Quinacrine**	5×10^{-4}	
Sodium diethyldithiocarbamate	1×10^{-3}	0
Bathocuproine	1×10^{-3}	0

*Cuvette contained: inhibitor in appropriate solvent; .02 M β-alanine-SO_4^{-2} buffer, pH 3.5; .2 M $FeSO_4$ in acid water; 0.1 ml of envelopes (0.2 mg protein).

**12% stimulation.

transport functions by reacting with biological membranes. The other inhibitors had little affect upon oxidation.

The envelope preparation represents a functional electron transport system where the electron from iron is moved along the chain to its ultimate acceptor oxygen. In the past, *in vitro* demonstrations of the "iron oxidase" have been difficult, and this report presents a clear-cut methodology involving low pH's where spontaneous iron oxidation is minimal. Figure 4 shows the envelope preparation.

Because of the technical problems associated with the oxidation catalyzed by these enzymes, our laboratory has attempted to study the "iron oxidase" system by using the intact electron transport chain located in cell envelopes. One approach has been to use temperature studies of iron oxidation with assays done over the range of 10°C to 70°C using both intact cells and cell envelopes. The data were then plotted as Arrhenius plots, and as Lineweaver-Burk plots to assess energy changes and kinetics properties of these enzymatic reactions.

Figure 5 shows the effect of temperature upon iron oxidation of both whole

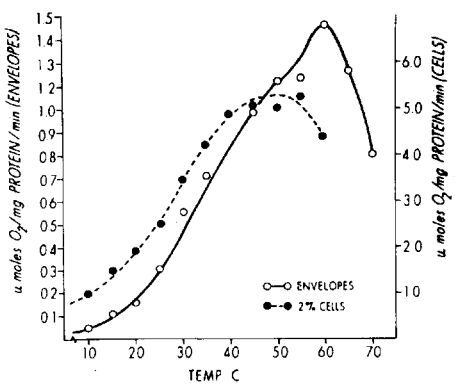


FIGURE 5. Temperature effects upon iron oxidation using cell envelopes and intact cells.

cells and cell envelopes. Oxidation rates increased with temperature until 60°C for cell envelopes, and then decreased rapidly. A sharper drop-off occurred with envelopes than with intact cells where a broader maximum temperature range was seen. An Arrhenius plot of these data are shown in figure 6 for cell

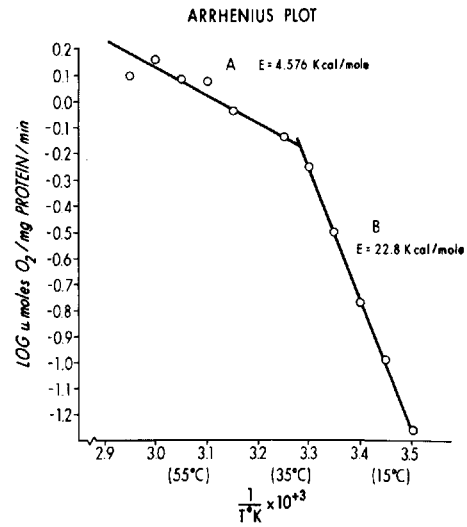


FIGURE 6. Temperature data for cell envelopes shown in Figure 5 plotted as an Arrhenius Plot. E = activation-energy or Arrhenius constant.

envelopes. A definitive break occurred in the curve at 35°C. The break represents a transition of the two curves and the slope of the lines are equal to an Arrhenius constant (or activation energy) for each of the enzyme reactions comprising the electron transport chain of "iron oxidase". Calculated values for

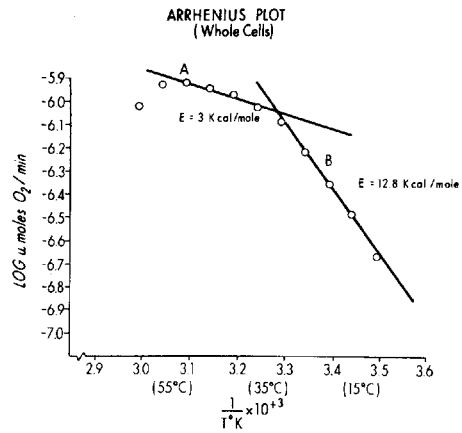


FIGURE 7. Temperature data for whole cells shown in Figure 5 plotted as an Arrhenius plot.

cell envelopes were 4.5 kcal/mole for cytochrome oxidase and 22.8 kcal/mole for cytochrome *c* reductase. Results of similar experiments with whole cells gave values of 3.0 and 12.8 kcal respectively for the two enzymes (figure 7). The relationship also was found at pH 2.5, with activation energies of 4.5 and 11.4 kcal determined for cytochrome oxidase and cytochrome *c* reductase respectively. At pH 4.5, no break in the linearity of the curve at 35°C was noted; however, at this pH oxidation rates were much slower and reproducibility was poor.

Figure 8 shows a series of K_m plots for apparent iron affinities to sites on envelopes at three pH's. In three separate experiments iron binding values fell in a fairly reproducible fashion with an increase in temperature at pH 3.5 whereas at the other two pH's changes in K_m were less over the temperature range examined. Generally these values are comparable to those of intact cells. For the moment there appears to be limited information that can be collected from such experiments. It is apparent, however, that the "iron oxidase" system remains intact for a limited period of time at higher temperatures and there is relatively little change in affinity. The maximum reaction rates (V_{max}) did increase with temperature as expected.

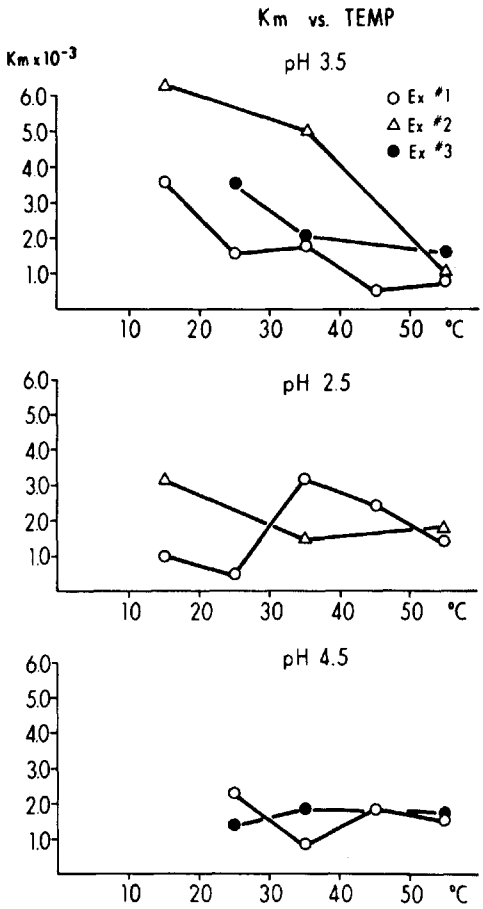


FIGURE 8. K_m values (M) plotted against temperature for three different pH's. The amount of Fe^{2+} added to the cuvette ranged from 7.5 to 40 μmol.

The work presented represents another component of information relative to biological iron oxidation. By chipping away at this process through research, the full story will be told. At that time, the design of preventative measures aimed at interferring with iron oxidation in nature will have more meaning.

Acknowledgments. Recognition of the contribution of experimental work reported here goes to Dr. Carl Bodo and George Barvinchak both of Syracuse University.

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